

ATTACHMENT III - PROTOCOL

Ecolab
Study Identification Number 1800073

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: CW32A Food Contact Sanitizing Efficacy

EPA REG. NO.: 1677-[pending]

ECOLAB GLP STUDY NUMBER: 1800073

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation **October 3, 2018**

Completion **December 31, 2018**

DESCRIPTION OF STUDY OBJECTIVE

CW32A (EPA Registration No. 1677-[pending]) will be tested to determine food contact surface sanitizing efficacy against *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229 with the test parameters outlined below. AOAC 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants will be the test method utilized in making the sanitizing claim.

Test Parameters

Ecolab SOP number:	MS009; <i>Germicidal & Detergent Sanitizing Action of Disinfectants</i>
Test Systems:	<i>Staphylococcus aureus</i> ATCC 6538 <i>Escherichia coli</i> ATCC 11229
Exposure Time:	30 seconds
Exposure Temperature:	25±1 °C
Test Substance Batches:	P081381 P081581 P081781
Test Substance Diluent:	500 ppm synthetic hard water
Test Substance Concentration:	0.25 oz/ gallon resulting in the active ingredient at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS).

TEST SUBSTANCE IDENTIFICATION

Test Substance Name: CW32A

Batch Identification: P081381
P081581
P081781

Formula Code: 919871

Date of Manufacture:

CW32A Batch Number	Date of Manufacture
P081381	August 13, 2018
P081581	August 15, 2018
P081781	August 17, 2018

An aliquot of the test substance batches will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, chemical quality verification in-life inspection, chemical quality verification data audit, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

Proposed QAU Monitoring

Protocol Audit
Chemical Quality Verification In-Life Inspection
Chemical Quality Verification Data Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

CHEMICAL QUALITY VERIFICATION

Proposed Experimental Start/Termination Dates

Experimental Start Date: October 2018

Experimental Termination Date: October 2018

Method

Chemical analysis was performed on each batch of the test substance concentrate to determine the concentration of the active ingredient under Ecolab GLP study number 1800060. Chemical analysis will be performed on a single batch of test substance use-solution prepared at 0.25 oz/gallon resulting in the active ingredient at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS) under this study number. The dilution procedure for the test substance use-solution chemical analysis can be found in the Test Substance Concentration section of the protocol. The test substance use-solution for the chemical analysis will be prepared in sterile laboratory purified water.

The chemical quality verification will be performed by the Analytical Lab using the method listed below. The method has been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance concentrate and the test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution sample.

QATM-216A; *Determination of Lactic Acid, Citric Acid, and Sodium Citrate by HPLC*
Lactic acid, citric acid, and/or citrate content are determined using HPLC-UV at 210 nm and external standard quantitation by peak area.

QATM-279; *Anionic Content by Surfactant Electrode*
The surfactant electrode responds to the concentration of anionic surfactant in aqueous solution. Using a standardized cationic solution as titrant and the surfactant electrode to identify the endpoint, the concentration of anionic surfactant in an aqueous solution can be determined by titration.

The most current QATMs and product specific Bill of Quality will be used during the course of this study for the chemical and physical analysis.

Interpretation of Results

The concentration of the active ingredient in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Concentrate Acceptance Limits		
Active Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Dodecylbenzene Sulfonic Acid (LAS)	11.4%	14.4%

The concentration of lactic acid in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Concentrate Acceptance Limits		
Ingredient	CSF Lower Certified Limit	CSF Upper Certified Limit
Lactic Acid	29.5%	38.6%

The concentration of the active ingredient in the test substance use-solution at the lower limit is < 1%. Therefore the lower acceptance limit will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

$$\begin{aligned} &\text{Calculated Lower Acceptance Limit for Dodecylbenzene Sulfonic Acid} \\ &= [0.0250\% - (0.0250 \times 0.1)] = 0.0225\% \end{aligned}$$

The calculated upper acceptance limit for the active ingredient in the test substance use-solution was determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

$$\begin{aligned} &\text{Calculated Upper Acceptance Limit for Dodecylbenzene Sulfonic Acid} \\ &= [0.0250\% + (0.0250 \times 0.02)] = 0.0255\% \end{aligned}$$

The concentration of the active ingredient in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limit of 0.0225-0.0255% Dodecylbenzene Sulfonic Acid.

The concentration of Lactic Acid in the test substance use-solution at the lower limit is < 1%. Therefore the lower acceptance limit will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

$$\begin{aligned} &\text{Calculated Lower Acceptance Limit for Lactic Acid} \\ &= [0.0648\% - (0.0648 \times 0.1)] = 0.0583\% \end{aligned}$$

The calculated upper acceptance limit for Lactic Acid in the test substance use-solution was determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

$$\begin{aligned} &\text{Calculated Upper Acceptance Limit for Lactic Acid} \\ &= [0.0648\% + (0.0648 \times 0.02)] = 0.0661\% \end{aligned}$$

The concentration of Lactic Acid in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limit of 0.0583-0.0661%.

The Chemical Quality Verification results will be reported in the final report of this study.

PESTICIDE EFFICACY TESTING

Proposed Experimental Start/Termination Dates

Experimental Start Date	October 2018
Experimental Termination Date	October 2018

Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS008	<i>Synthetic Hard Water Preparation & Standardization</i>
MS009	<i>Germicidal & Detergent Sanitizing Action of Disinfectants</i>
MS088	<i>Test Substance Use-Solution Preparation for Analysis</i>

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of a non-halide food contact surface sanitizer; three samples, representing different batches are required to be tested. The required organisms are *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229. AOAC 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants for the above stated organisms are recommended based on the U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2300: Sanitizers for Use on Hard Surfaces– Efficacy Data Recommendations, September 04, 2012. Also, U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP MS009; *Germicidal & Detergent Sanitizing Action of Disinfectants* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229. Identification will be performed by observing the colony morphology and performing a Gram stain.

Statement of Proposed Statistical Method

None

Test Substance Diluent

500 ppm synthetic hard water prepared as described in Ecolab Microbiological Services SOP MS008; *Synthetic Hard Water Preparation & Standardization* will be the diluent.

Test Substance Concentration

Antimicrobial efficacy testing will be performed with CW32A diluted at 0.25 oz/gallon to at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid (LAS).

Active Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Dodecylbenzene Sulfonic Acid (LAS)	11.4%	1.126	0.195%	250 ppm

*Study proposed dilution: (0.25 oz/1 gallon) x (1 gallon/128 oz) x (100%) = 0.195%

Resulting ppm of active ingredient =

$$\left(\frac{\% \text{Active at LCL}}{100\%} \right) \left(\frac{\% \text{Dilution}}{100\%} \right) (\text{Specific Gravity}) (10^6)$$

The following calculation will be used to ensure that the active ingredient is at or below the lower limit of 250 ppm Dodecylbenzene Sulfonic Acid in the test substance use-solution:

Dilution based on % active from QATM-279 analysis:

$$\text{g of test substance batch in 600 g to yield 250 ppm Dodecylbenzene Sulfonic Acid} = \frac{(250 \text{ ppm})(600 \text{ g})(100\%)}{(10^6) (\% \text{ Active in batch})}$$

Antimicrobial efficacy testing will be performed with CW32A diluted at 0.25 oz/gallon to at or below the lower limit of 648 ppm Lactic Acid.

Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Lactic Acid	29.5%	1.126	0.195%	648 ppm

*Study proposed dilution: (0.25 oz/1 gallon) x (1 gallon/128 oz) x (100%) = 0.195%

Resulting ppm of active ingredient =

$$\left(\frac{\% \text{Active at LCL}}{100\%} \right) \left(\frac{\% \text{Dilution}}{100\%} \right) (\text{Specific Gravity}) (10^6)$$

The following calculation will be used to ensure that Lactic Acid is at or below the lower limit of 648 ppm in the test substance use-solution:

Dilution based on % active from QATM-216A analysis:

$$\text{g of test substance batch in 600 g to yield 648 ppm Lactic Acid} = \frac{(648 \text{ ppm})(600 \text{ g})(100\%)}{(10^6) (\% \text{ Active in batch})}$$

The test substance use-solutions should be prepared as shown below in bold or with an equivalent dilution to ensure both the Dodecylbenzene Sulfonic Acid (LAS) and the Lactic Acid are at or below their lower limits for use in efficacy testing:

CW32A Batch Number	Concentration from Analysis	Test Substance Weight*: Diluent Weight*
P081381	% Anionic (QATM 279): 12.9%	1.16 g : 598.84 g
	% Lactic Acid (QATM 216A): 33.3%	1.17 g : 598.83 g
P081581	% Anionic (QATM 279): 12.9%	1.16 g : 598.84 g
	% Lactic Acid (QATM 216A): 33.8%	1.15 g : 598.85 g
P081781	% Anionic (QATM 279): 12.9%	1.16 g : 598.84 g
	% Lactic Acid (QATM 216A): 33.8%	1.15 g : 598.85 g

Chemical analysis was performed under Ecolab GLP study number 1800060.

*Weights may vary by $\pm 0.03\text{g}$.

Exposure Time/Temperature

The test systems will be exposed to the test substance for 30 seconds at $25 \pm 1^\circ\text{C}$

Neutralizer Medium

9 mL DE Broth

Plating Medium

Tryptone Glucose Extract Agar

Incubation Time/Temperature

All plates are incubated for 24-30 hours at $35 \pm 2^\circ\text{C}$

Test Controls

The following controls will be incorporated with the test procedure for each test system:

- a. Initial Numbers Control
- b. Neutralization Control
- c. Test System Purity
- d. Test Substance Diluent Sterility Control
- e. Test Substance Sterility Control

Details on each of the above controls can be found in Ecolab SOP MS009 located in Protocol Appendix.

Interpretation of Test Results

The performance standard for a food contact sanitizer requires at least a 5 log reduction ($\geq 99.999\%$) in the numbers of both *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229 compared to the initial numbers control results within 30 seconds.

DATA RETENTION

Following the completion of the study, the original final report and raw data will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

- **Name and Address of Sponsor**

Kaitlin Lake
Ecolab Schuman Campus
655 Lone Oak Drive
Eagan, MN 55121

- **Name and Address of Performing Laboratory**

Ecolab Schuman Campus
655 Lone Oak Drive
Eagan, MN 55121

- **Name and Address of Study Director**

Laurinda Holen
Ecolab Schuman Campus
655 Lone Oak Drive
Eagan, MN 55121

Kaitlin Lake
Sponsor
Laurinda Holen
Study Director

10.3.18
Date
10/3/18
Date

PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS008	<i>Synthetic Hard Water Preparation & Standardization</i>
MS009	<i>Germicidal & Detergent Sanitizing Action of Disinfectants</i>
MS088	<i>Test Substance Use-Solution Preparation for Analysis</i>

**ECOLAB
MICROBIOLOGICAL SERVICES**

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

EFFECTIVE: 04/01/16

1.0 PURPOSE

To describe how to prepare standardized synthetic hard water solution to be used for diluting products that possess hard water claims.

2.0 SYNTHETIC HARD WATER PREPARATION

2.1 Fill out a media preparation sheet for Solution A and Solution B. Retain in the Media Preparation Log Book. Prepare 1 L of each solution or alternate amount with proportional ingredients.

2.2 Solution A Preparation

Magnesium Chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$)	67.74 \pm .1 g
Calcium Chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	97.99 \pm .1 g
Sterile Lab Purified Water	1 L

2.2.1 Dissolve powders in 600 mL of boiled lab purified water, and then bring to 1 L volume in a 1 L volumetric flask after solution has cooled.

2.2.2 Dispense into appropriate containers (for example, 250 mL Pyrex screw cap bottles) and autoclave for ≥ 15 minutes at $\geq 121^\circ\text{C}$.

2.2.3 Label using the standard Ecolab labels with a 1 month expiration date and store at $2 - 8^\circ\text{C}$.

2.2.4 Quality Control

2.2.4.1 Visual: Clear solution

2.2.4.2 Sterility Check: Sterile after incubation at $32 \pm 2^\circ\text{C}$ for ≥ 5 days

2.2.4.3 Expiration Date: One month at $2 - 8^\circ\text{C}$

2.3 Solution B Preparation

Sodium Bicarbonate (NaHCO_3)	56.03 \pm .1 g
Sterile Lab Purified Water	1 L

SA 10/31/18
EXACT
COPY

Standard Operating Procedure

Ecolab Controlled Document

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

- 2.3.1 Dissolve in 600 mL of boiled lab purified water, then bring to 1 L volume in a 1 L volumetric flask with lab purified water after solution has cooled.
- 2.3.2 Filter sterilize through a 0.45 micron filter into appropriate sterile containers. (approximately 150 – 200 mL per container)
- 2.3.3 Label using the standard Ecolab labels with a one month expiration date and store at 2 – 8°C.
- 2.3.4 Quality Control
- 2.3.4.1 Visual: Clear solution
- 2.3.4.2 Sterility Check: Sterile after incubation at $32 \pm 2^\circ \text{C}$ for ≥ 5 days
- 2.3.4.3 Expiration Date: One month at 2 – 8°C
- 2.4 Hard Water Preparation
- 2.4.1 To avoid precipitation of the hard water solution, water should be at room temperature before the addition of Solutions A or Solution B.
- Total hardness as ppm $\text{CaCO}_3 = 2.495 \times \text{ppm Ca} + 4.115 \times \text{ppm Mg}$
- 2.4.2 To each 1 L of water to be prepared add 1 mL of Solution A for each 100 ppm of CaCO_3 hardness desired plus 4 mL of Solution B (e.g. for 500 ppm synthetic hard water add 5 mL of Solution A and 4 mL of Solution B per liter of water).
- 2.4.3 Bring to 1 L volume with sterile lab purified water. If preparing more than 1 L, combine flasks in a sterile 4 L beaker blender after adding appropriate amounts of Solutions A and Solution B and bringing to volume.
- 2.5 Alternate Hard Water Preparation: Commercial Preparation
- 2.5.1 Use a commercially available standard, preferably NIST traceable, to prepare synthetic hard water (e.g. Hach Chemical Company 218710).
- 2.5.2 To prepare a 400 ppm as CaCO_3 solution, add four ampules of 10,000 ppm as CaCO_3 standard (10 mL each ampule) to a 1 L volumetric flask.
- 2.5.3 Add sterile lab purified water up to 1 L mark. Solutions of other water hardness and different volumes may be prepared as appropriate.
- 2.6 The pH of all test waters less than 2000 ppm hardness (as CaCO_3) should be 7.6 – 8.0. Adjustment of hard water pH using NaOH or HCl may be necessary depending on the starting water pH.

Ecolab Controlled Document

Standard Operating Procedure

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

3.0 STANDARDIZATION OF SYNTHETIC HARD WATER

3.1 Method Check – Prior to standardization of the synthetic hard water, the accuracy of the titration method must be checked by analyzing a 500 ppm CaCO_3 standard. This must be performed on a monthly basis or when testing new batches of Solution A and Solution B.

3.1.1 Dilute 10 mL of a 1000 ppm CaCO_3 standard (1 mL = 1 mg CaCO_3) in 10 mL of lab purified water to result in a 500 ppm CaCO_3 solution.

3.1.2 Dilute 10 mL of the 500 ppm CaCO_3 solution in 40 mL of lab purified water in a beaker.

3.1.3 Test solution as described in 3.2.2 – 3.2.5.

3.1.4 The hardness of the 500 ppm solution is determined as follows:

$$\text{hardness (ppm)} = (\text{mL EDTA}) \times 100$$

3.1.5 Record the result and the lot number of the standard on Form 3011. Hardness of the 500 ppm CaCO_3 solution must be 500 ± 20 ppm CaCO_3 . Failure of the standard to fall within this range indicates a problem in the test method. Corrective actions should be documented in the comments section on Form 3011. The procedure may be used for standardization of synthetic hard water only when results of the standard are within the range described above.

3.1.6 Records from the current and previous year will be kept in the Microbiological Services Equipment Maintenance binder. All earlier records will be archived in the first quarter of the current year. For example, records from 2016 will be archived by March of 2018. Records will be transferred to Ecolab Archives at the Ecolab Schuman Campus in Eagan, MN or to an approved off-site location.

3.2 Sample Testing/Standardization

3.2.1 Dilute 10 mL of prepared hard water in 40 mL of lab purified water in a beaker.

3.2.2 Add 1 mL water hardness buffer with magnesium. Use hood when adding; the buffer has irritating vapors.

3.2.2.1 The buffer is VWR product code VW3491 (or equivalent)

08/10/18
31

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

3.2.2.2 Approximate composition of buffer, % by weight:

Ammonia	56-57
Ammonium chloride	6-7
EDTA-Magnesium Tetraacetate Salt	0.5
Water	> 35

Note: This buffer has a relatively short expiration.

- 3.2.3 Optional: Add 1 mL inhibitor – needed only if previous titration without it has been unsatisfactory (refer to 3.2.5.2).
- 3.2.4 Add just enough Ecolab hardness indicator #016 to yield a pink coloration upon dissolving.
- 3.2.4.1 Hardness indicator 016 contains Calgamite (1-(1-hydroxy-4-methyl-2-phenylazo)-2-naphthol-4-sulfonic acid) as the actual indicator, along with inert ingredients.
- 3.2.4.2 It is obtained from Ecolab Test Kits (order through F&B Customer Service) at the Ecolab Engineering Center.
- 3.2.5 Add 0.01M EDTA slowly until the pink coloration turns blue. Record the number of milliliters of EDTA needed to create the color change.
- 3.2.5.1 The titration should be completed within five minutes of buffer addition to minimize tendency toward CaCO_3 precipitation.
- 3.2.5.2 If the end point color change is not clear and sharp (e.g. the color changes to blue and then drifts back to pink) then an inhibitor/complexing agent must be added (or possibly, the indicator has deteriorated).
- 3.2.5.3 Prepare inhibitor solution by dissolving 5.0 g sodium sulfide nonahydrate ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$) or 3.7 g $\text{Na}_2\text{S} \cdot 5\text{H}_2\text{O}$ in 100 mL distilled water. Prepare and dispense in hood. This inhibitor solution deteriorates quickly though air oxidation and should be made each day it is needed.
- 3.2.5.4 Dilute new sample of test solution and re-titrate beginning with step 3.2.2, including addition of inhibitor.

Ecolab Controlled Document

Standard Operating Procedure

9/11/2018

TITLE: Synthetic Hard Water Preparation & Standardization

NUMBER: MS008-25

3.2.6 The hardness of the water is determined as follows:

$$\begin{aligned}\text{Hardness as mg CaCO}_3/\text{L} &= (\text{mL EDTA} \times 1000)/10 \text{ mL of sample} \\ &= \text{mL EDTA} \times 100\end{aligned}$$

3.2.7 Upon titration, hardness must not exceed 20 ppm above or below the ppm specified in test procedure/protocol/lab statement. Therefore, if a claim is for 500 ppm, the titration must yield 500 ± 20 ppm. If ppm hardness is out of the established range, the sample should be retitrated. Upon a second titration, if ppm hardness is still outside established ranges, the hard water must be diluted or additional solution added to yield the desired ppm. After adjustments have been made, the water must be titrated to determine ppm hardness.

3.2.8 Only two adjustments may be made to the hard water following the above procedure. If the hard water is outside the established limits after two adjustments, the water must be disposed of and the process reinitiated.

3.2.7 For GLP testing, record Hard Water Preparation and Standardization on Form 3010 or Form 3113.

4.0 RELATED FORMS

- 4.1 Form 3010: Synthetic Hard Water Preparation & Standardization
- 4.2 Form 3011: Water Hardness Standard Results
- 4.3 Form 3072: Solution A Prep Log
- 4.4 Form 3074: Solution B Prep Log
- 4.5 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

- 5.1 AOAC (2011) Method 960.09 (E)
- 5.2 APHA, Standard Methods for the Examination of Water & Wastewater, 21st Ed., 2005. Section 3500-Ca B. EDTA Titrimetric Method.

6.0 MOST RECENT REVISION SUMMARY

In 2.1, added option to prepare amount other than 1 L of Solution A or Solution B.

0110318
JAN
2016

Prepared by: Yindra Miguere Date: 3/3/2016
Quality Assurance: [Signature] Date: 03 Mar 2016
Management: Shirley St. Clair Date: 03 Mar 2016

**ECOLAB
MICROBIOLOGICAL SERVICES**

Standard Operating Procedure

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

EFFECTIVE: 10/19/17

1.0 PURPOSE

To determine the efficacy of products used for sanitizing pre-cleaned, nonporous food contact surfaces. Additionally, this document describes the procedure to determine the efficacy of teat dips. The MS009 Attachment details the modifications required for teat dip efficacy testing.

2.0 CULTURE MEDIA – Propagation

- 2.1 AOAC Nutrient Broth
- 2.2 Nutrient Agar A (slants not made from pre-mixed dehydrated media)
- 2.3 Nutrient Agar B (plates)
- 2.4 Other media suitable for culturing specified test systems

3.0 SUBCULTURE MEDIA – Plating

- 3.1 Tryptone Glucose Extract Agar
- 3.2 D/E Agar
- 3.3 Brain Heart Infusion Agar
- 3.4 Tryptic Soy Agar (with or without 5% Sheep's Blood)
- 3.5 Other media suitable for culturing specified test systems

4.0 NEUTRALIZER

- 4.1 D/E Neutralizing Broth
- 4.2 Chambers Medium
- 4.3 0.1 – 0.5% Sodium thiosulfate
- 4.4 Lethen Broth
- 4.5 Other appropriate neutralizer

5.0 REAGENTS & APPARATUS

- 5.1 Phosphate Buffered Dilution Water (PBDW)
- 5.2 Phosphate Buffered Saline with 0.1% (v/v) Tween 80 (PBS & Tween 80)

Handwritten: 11/13/18
1800073

Ecolab Controlled Document

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

5.3 Glassware

- 5.3.1 Sterile 250 mL Erlenmeyer flasks
- 5.3.2 Appropriately sized volumetric flasks
- 5.3.3 20 × 150 and 25 × 150 mm test tubes

5.4 Petri Dishes

- 5.4.1 Sterile disposable Petri dishes, 15 × 100 mm

5.5 Water Bath

- 5.5.1 Constant temperature water bath that can maintain a test temperature ± 1°C of required test temperature

5.6 Transfer Loops

- 5.6.1 Reusable metal or sterile plastic disposal transfer loops

5.7 Sterile Buchner Funnel (or equivalent) containing Whatman No. 2 Filter Paper

5.8 Sterile disposable 50 mL centrifuge tubes

5.9 Pipets/Transfer Device

- 5.9.1 Sterile disposable pipets
- 5.9.2 Micropipettor with sterile disposable tips

6.0 TEST SUBSTANCE

- 6.1 To test hard water tolerance, the test substance may be diluted in synthetic hard water (refer to MS008).
- 6.2 If dilution of the test substance is required, use greater than 1.0 mL or 1.0 g of product. The use-solution must be tested within three hours of preparation or within the known stability of the solution.

7.0 TEST SYSTEMS PREPARATION

7.1 Test Systems

- 7.1.1 *Staphylococcus aureus* ATCC 6538
- 7.1.2 *Escherichia coli* ATCC 11229
- 7.1.3 Refer to the MS009 Attachment for additional microorganisms that may be used to evaluate teat dip efficacy
- 7.1.4 Other test systems may be tested with appropriate culturing modifications

Ecolab Controlled Document

Standard Operating Procedure

Handwritten signature
10/23/18

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

- 7.2 Defrost a single cryovial of frozen stock culture at room temperature and briefly vortex. Streak one loopful of the thawed frozen stock onto a Nutrient Agar A Slant and incubate at $35 \pm 2^{\circ}\text{C}$ for 24 ± 2 hours.
- 7.3 For the final test culture, add 5 mL PBDW to the inoculated slant. Use a sterile loop to dislodge growth from agar surface. Collect mixture and transfer to the balance of the 99 mL PBDW and mix. Inoculate a minimum of five Nutrient Agar B plates with 200 μL of the mixture to create a bacterial lawn. Incubate at $35 \pm 2^{\circ}\text{C}$ for 24 ± 2 hours.
- 7.4 Harvest the test system from the plates by adding a minimum of 5 mL of PBS + Tween 80 to each plate. It may be necessary to use less than 5 mL of PBS + Tween 80 to harvest some test systems in order to achieve the necessary titer. Use a sterile rod or equivalent to gently dislodge the culture from the agar surface. Combine the culture from all plates and mix thoroughly.
- 7.5 Filter the culture through sterile Whatman No. 2 filter paper contained in Buchner funnel (or equivalent). To accomplish this, place a sterile tube in the collection flask and assemble the Buchner funnel on top. Pre-wet the filter paper with about 1 mL of PBDW and initiate the vacuum to create a proper seal. Process the culture directly through the filter paper, under vacuum, collecting the test system suspension into the sterile tube. Once the culture is processed, remove the tube and vortex the suspension.
 - 7.5.1 Adjust the density of the culture suspension, if necessary, by dilution using sterile PBDW to yield approximately $1.0 \times 10^9 - 1.0 \times 10^{10}$ organisms per milliliter.
 - 7.5.2 1.0×10^9 organisms/mL corresponds roughly to % transmittance readings of 0.1% to 1.0%T at 580 nm.
 - 7.5.3 Adjusting the density of the culture suspension may not be required for determining the efficacy of test dips.

8.0 OPERATING TECHNIQUE

- 8.1 Refer to the MS009 Attachment for the operating technique suggested for determining the efficacy of test dips.
- 8.2 Dispense 99 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare a single flask for each test substance to be tested. Duplicate flasks should be evaluated during non-regulated or screening tests, where possible.
- 8.3 Also prepare a flask with 99 mL of sterile PBDW, per test system, for enumeration of initial numbers control and treat in the same manner as the test flasks.

08/10/2018
607

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

- 8.4 Place flasks into a $25 \pm 1^\circ\text{C}$ temperature water bath (or other desired test temperature) and let equilibrate for ≥ 10 minutes.
- 8.5 Adding Test System to Test Substance
- 8.5.1 Swirl the test flasks, creating enough residual motion to prevent pooling of the test system.
- 8.5.2 While the liquid is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance midway between the center and edge of the flask. Add 1 mL of test system suspension to 99 mL of the test substance.
- 8.5.3 Avoid touching the sides of the flask with the pipet.
- 8.5.4 After addition of the inoculum, swirl the flask to thoroughly mix contents.
- 8.5.5 At the 30 second exposure period (or other exposure time(s) as appropriate), transfer a 1 mL portion of the test mixture to 9 mL of appropriate neutralizer and mix well. This corresponds to the 10^{-1} dilution. If necessary to achieve neutralization, a 99 mL aliquot of neutralizer may be used. This corresponds to the 10^{-2} dilution.
- 8.6 Plate 1 mL and 0.1 mL of the neutralized contents in quadruplicate. Single plating and additional dilutions may be performed if testing is not for regulatory purposes. Use the pour plate or spread plate technique and an appropriate subculture medium. For calculation purposes, when 9 mL of neutralizer is used, this corresponds to the 10^{-1} and 10^{-2} dilutions, respectively.
- 8.7 For the numbers control, add 1 mL of the test system suspension to 99 mL of PBDW in the same manner as done in the test. Within 30 seconds of addition of test system suspension, transfer 1 mL into 9 mL neutralizer (or 99 mL if used in the test) and mix well. This corresponds to the 10^{-1} or 10^{-2} dilution, respectively.
- 8.8 Make serial 10-fold dilutions in 9 mL of PBDW to 10^{-6} .
- 8.9 Plate 1 mL and 0.1 mL of the 10^{-6} dilution in quadruplicate using pour or spread plate technique using the subculture medium used in the test. For calculation purposes, this corresponds to the 10^{-6} and 10^{-7} dilutions, respectively.

Note: 1 mL aliquots may be split in half and plated over two plates.

Ecolab Controlled Document

Standard Operating Procedure

Handwritten signature and date:
10/23/18

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

9.0 CONTROLS

9.1 Neutralization Control

- 9.1.1 A neutralization confirmation test must be performed in advance or concurrently with the efficacy test.
- 9.1.2 Serially dilute the test system suspension to target between 10 – 100 CFU once the control is plated. When the target titer is 10^9 to 10^{10} , the 10^{-5} and 10^{-6} dilutions should provide an average of 10 – 100 CFU, once plated. Alternative dilutions may be used where appropriate.
- 9.1.3 Add 0.1 mL of the diluted test system suspension to inoculate each of the control tests in 9.1.4 and mix thoroughly. Test A should be inoculated within 30 seconds of preparation. After inoculation, each of the tests are held for a minimum of two minutes prior to plating. Plate 0.1 mL and 1.0 mL in duplicate. Use the pour plate or spread plate technique and the subculture medium used in the test.
- 9.1.4 Prepare the neutralizer control tests as shown in the table below:

Control Test	Description
A (Neutralizer Confirmation or NCT)	1 mL test substance to 9 or 99 mL neutralizer
B (Neutralizer Toxicity Treatment or NTT)	10 or 100 mL neutralizer
C (Test Culture Titer or TCT)	10 mL or 100 mL PBDW

9.2 Test System Purity

- 9.2.1 Inoculate the test system suspension onto Tryptic Soy agar with 5% sheep blood (e.g. Blood Agar plate) and streak for isolated colonies. If the test system does not grow on Blood Agar, use an alternate agar medium that supports growth.
- 9.2.2 Gram stain the test system.

9.3 Diluent Sterility

- 9.3.1 Plate 1.0 mL of the diluent using pour plate or spread plate technique.

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

9.4 Test Substance Sterility

9.4.1 Plate 1.0 mL of the test substance using pour plate or spread plate technique.

Note: Neutralizer and PBDW sterility testing is conducted with routine media QC procedures.

10.0 INCUBATION

10.1 For regulated studies, incubate plates of *S. aureus* and *E. coli* at $35 \pm 2^\circ\text{C}$ for 24 – 30 hours.

10.2 For screening studies, incubate plates of *S. aureus* and *E. coli* at $35 \pm 2^\circ\text{C}$ for 2 – 3 days. Extended incubation helps to confirm the presence of sub-lethally injured cells that require extended incubation for recovery.

10.3 Incubate plates of other microorganisms at a time and temperature that provides adequate growth (e.g. 48 ± 4 hours).

11.0 DATA ANALYSIS

11.1 Enumerate and record plate counts as Colony Forming Units (CFU)/plate.

11.2 For initial numbers control and efficacy survivor counts determine the average CFU/mL as follows:

$$\text{Average CFU/mL} = \frac{(\text{Average CFU for } 10^{-x}) + (\text{Average CFU for } 10^{-y})}{10^{-x} + 10^{-y}}$$

Where 10^{-x} and 10^{-y} are the dilutions plated.

Use counts of 0 – 300 for calculation purposes. Score > 300 as TNTC (too numerous to count). If the average CFU/plate is < 1, then use < 1 when calculating the average CFU/mL.

11.3 Calculate mean \log_{10} density for numbers controls plates. Calculate the mean \log_{10} density for the treated sample plates. Calculate the \log_{10} reduction for the treated sample:

\log_{10} reduction = mean \log_{10} numbers control – mean \log_{10} treated sample

Note: If duplicate flasks are tested, average the CFU/mL results for each flask to obtain a final average log reduction.

Ecolab Controlled Document

Standard Operating Procedure

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

- 11.4 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control mean \log_{10} density value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standard and the numbers control mean \log_{10} density is below 7.0, no testing is necessary.
- 11.5 In order to demonstrate effective neutralization of the sanitizer, the difference of A or B from C must be no more than 1.0 log.
- 11.6 Test system purity should have typical morphology for each of the organisms tested:
- 11.6.1 *Staphylococcus aureus* ATCC 6538: Medium to large, convex, circular, glistening, smooth, creamy, opaque, beta hemolytic – both light gold and darker gold colonies may be present on Blood Agar.
- 11.6.2 *Escherichia coli* ATCC 11229: Large, irregular, raised, gray and rough; greening of agar may be present on Blood Agar.
- 11.7 The diluent sterility control and test substance sterility control passes if there is no growth on the agar plate.

12.0 PERFORMANCE CRITERIA

- 12.1 In order for a sanitizer to be deemed effective, a 5 log reduction in the count of the number of microbes within 30 seconds is necessary. All test controls must also be valid.

13.0 RELATED FORMS

- 13.1 Form 3012: Germicidal & Detergent Sanitizing Action of Disinfectants
13.2 Form 3114: Germicidal & Detergent Sanitizing Action of Disinfectants – Teat Dip Bench Sheet

14.0 REFERENCES

- 14.1 MS008: Synthetic Hard Water Preparation & Standardization
14.2 MS040: Media Preparation & Storage – Media & Chemicals
14.3 Created from AOAC Method 960.09; Germicidal & Detergent Sanitizing Action of Disinfectants, 2013
14.4 EPA Good Laboratory Practice Standards, 40 CFR Part 160
14.5 US EPA OCSPP 810.2300: Sanitizers for Use on Hard Surfaces – Efficacy Data Recommendations, September 4, 2012

RA 10/31/18

TITLE: Germicidal & Detergent Sanitizing Action of Disinfectants

NUMBER: MS009-26

15.0 MOST RECENT REVISION SUMMARY

General clarifications/text revisions added to 5.4.1, 7.1.4, 7.4, 8.5.4, 8.5.5, 8.6, 8.7, 8.9, 9.1 (title), 9.1.3, 9.4.1, 10.1 and 11.5. Added TSA/BAP to section 3.0. Revised 7.5 to clarify test organism procedure. Changed 8.2 to require the use of one flask for regulated testing and recommend two flasks for non-regulated/screening tests. Added note about splitting 1 mL aliquots after 8.9. Clarified text in 9.1.2 to recommend the 10-5 and 10-6 dilutions. Added note to 9.4. Added 10.2 (incubation of R&D studies for 2 –3 days). Added note in 11.3 to clarify how to calculate results from two flasks.

Ecolab Controlled Document

Standard Operating Procedure

Prepared by: [Signature] Date: 10-18-17
Quality Assurance: [Signature] Date: 18 Oct 2017
Management: Shirley St. Clair Date: 18 Oct 2017

10/13/18

MS009 Attachment – 10/19/17

1.0 PURPOSE

To describe the modifications to MS009 required for determining the efficacy of teat dips.

2.0 TEST SYSTEMS

2.1 In addition to the microorganisms listed in step 7.1 in MS009, the following may be used for teat dip efficacy testing:

- *Streptococcus uberis* ATCC 27958
- *Streptococcus agalactiae* ATCC 27956
- *Streptococcus dysgalactiae* ATCC 27957
- *Pseudomonas aeruginosa* ATCC 15442
- *Klebsiella pneumoniae* ATCC 4352
- *Enterobacter aerogenes* ATCC 13048

3.0 OPERATING TECHNIQUE FOR TEAT DIP EFFICACY TESTING

3.1 Operating Technique without Milk (typically for pre-milking treatments)

3.1.1 Dispense 99 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare duplicate flasks for each test substance and test system combination to be tested. Also prepare a flask with 99 mL of sterile PBDW for enumeration of initial numbers and treat in the same manner as the test flasks.

3.1.2 Swirl a test flask. While the test substance is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance midway between the center and edge of the flask. Add 1 mL of the test system suspension to 99 mL of the test substance. Avoid touching the sides of the flask with the pipet. Swirl the flask to thoroughly mix contents. After a 30 second exposure period, transfer 1 mL to 9 or 99 mL (or other volume as appropriate) of the appropriate neutralizer (based on inactivation of the test substance) and mix well immediately. Other exposure periods (e.g. 15 seconds) may be used. Because duplicate flasks are tested, duplicate plating of the test flasks is an acceptable alternative to quadruplicate plating.

Note: If the viscosity of the test substance does not allow for even distribution of the test system upon inoculation, the test flask may be swirled continuously throughout the exposure period. Alternately, a stir bar and stir plate may be used to aid in the distribution of the test system.

3.1.3 Enumerate microorganisms surviving treatment and initial numbers control as described in steps 8.7 – 8.9 in MS009.

MS009 Attachment – 10/19/17

- 3.1.4 Perform the neutralization controls as described in steps 9.1.1 – 9.1.4 in MS009.
- 3.1.5 Perform the test system purity control, diluent sterility control (if applicable) and the test substance sterility control as described in steps 9.2, 9.3 and 9.4 respectively in MS009.
- 3.1.6 Incubate plates as described in step 10.0 in MS009.
- 3.1.7 Perform data analysis as described in steps 11.1 – 11.3 in MS009.
- 3.1.8 For results to be considered efficacious, a 4 log reduction in the numbers of *Escherichia coli* and *Staphylococcus aureus* must occur within a 30 second exposure without milk.
- 3.1.9 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standards and the number control mean \log_{10} density is below 7.0, no retesting is necessary.
- 3.1.10 Neutralization control, test system purity control and diluent sterility control analysis should be performed as described in steps 11.5, 11.6 and 11.7 in MS009.
- 3.2 Operating Technique with Milk (typically for post-milking treatments)
- 3.2.1 Dispense 90 mL of test substance into a sterile 250 mL Erlenmeyer flask. Prepare duplicate flasks for each test substance and test system combination to be tested. Also prepare a flask with 99 mL of sterile PBDW for enumeration of initial numbers and treat in the same manner as the test flasks.
- 3.2.2 Swirl a test flask. Add 10 mL of sterile milk, swirl well and remove 1 mL for a total of 99 mL in the test flask. This gives a 10% milk challenge. Immediately, swirl the test flask and, while the liquid is still in motion, place the tip of the pipet containing the test system so it is partially immersed in the test substance/milk mixture midway between the center and edge of the flask. Add 1 mL of culture to 99 mL of the test substance/milk mixture. Avoid touching the sides of the flask with the pipet. Swirl the flask to thoroughly mix contents. After a 30 second exposure period, transfer 1 mL to 9 or 99 mL (or other volume as appropriate) of the appropriate neutralizer (based on inactivation of the test substance) and mix well immediately.
- Other exposure periods (e.g. 15 seconds) may be used.

Ecolab Controlled Document

Standard Operating Procedure

MS009 Attachment – 10/19/17

Note: If the viscosity of the test substance does not allow for even distribution of the test system upon inoculation, the test flask may be swirled continuously throughout the exposure period. Alternately, a stir bar and stir plate may be used to aid in the distribution of the test system.

- 3.2.3 Enumerate microorganisms surviving treatment and initial numbers control as described in steps 8.7 – 8.9 in MS009.
- 3.2.4 Perform the neutralization controls as described in steps 9.1.1 – 9.1.4 in MS009.
- 3.2.5 Perform the test system purity control, diluent sterility control (if applicable) and the test substance sterility control as described in steps 9.2, 9.3 and 9.4 respectively in MS009.
- 3.2.6 Plate 1.0 mL milk using pour plate or spread plate technique. Milk sterility control passes if there is no growth on the milk sterility agar plate.
- 3.2.7 Incubate plates as described in step 10.0 in MS009.
- 3.2.8 Perform data analysis as described in steps 11.1 – 11.3 in MS009.
- 3.2.9 For results to be considered efficacious, a 4 log reduction in the numbers of the test system must be achieved in the exposure time with a milk challenge.
- 3.2.10 For the test to be considered valid, the numbers control must fall between 7.0 – 8.0 logs. For tests where the product meets the performance standard and the numbers control value is above 8.0, no retesting is necessary. For tests where the product fails to meet the performance standards and the number control mean \log_{10} density is below 7.0, no retesting is necessary.
- 3.2.11 Neutralization control, test system purity control, diluent sterility control and test substance sterility control analysis should be performed as described in steps 11.5, 11.6 and 11.7 in MS009.

10/10/18
[Signature]

5

ECOLAB
MICROBIOLOGICAL SERVICES

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

EFFECTIVE: 06/15/18

1.0 PURPOSE

To describe the preparation and active ingredient analysis of a diluted test substance (test substance use-solution). Use-solution analysis is included with pesticide efficacy studies, chemical quality verification studies and contract lab studies to verify that the active ingredient concentration in the use-solution corresponds to the dilution made for the claimed active ingredient(s).

2.0 PROCEDURE

2.1 Typically, use-solutions are prepared as follows –

2.1.1 Use-solutions are prepared at the Lower Certified Limit (LCL) or lower limit for efficacy studies.

2.1.1.1 The Lower Certified Limit refers to the lower level of active ingredient in the formulation (e.g. the concentrate or ready-to-use formula).

2.1.1.2 The Lower Limit refers to the lower level of active ingredient in the use-solution after a concentrate is adjusted.

2.2 Determine the concentration of active ingredient in the test substance concentrate to verify it is within claimed limits. Perform the analysis for each active ingredient in the product.

2.3 Prepare the test substance use-solution according to label instructions or as specified in protocol using diluent as described in 2.4. This use-solution should be labeled according to M032.

2.4 Deionized water may be used as the test substance diluent or the test substance diluent (e.g. hard/soft water or label instructed diluent) may be prepared in the same manner as used for pesticide efficacy testing.

Example: A 1:64 dilution is 1 part test substance, 63 parts diluent.

2.5 Analyze the test substance use-solution for active ingredient concentration using the same validated QATM that is, or will be, included in the finished good Bill of Quality (BOQ).

06/10/18

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

Note: The method used to measure active ingredient concentration in the use-solution may have limited sensitivity, accuracy and precision for quantitating the minimal levels of active ingredient found in many use-solutions. These factors may need to be considered when interpreting results. Any modifications to the QATM to adjust for this should be specified in the protocol.

- 2.6 Analyze the results. The active ingredient concentration in the use-solution should fall within the acceptance range as outlined in 3.5.

3.0 Formulas to Determine Use-solution Amounts and Acceptance Criteria

3.1 Dilution Factor (DF) Determination

3.1.1 Dilution Factor by Volume (DF_{vol})

$$\text{Example: Dilution Factor (DF}_{\text{vol}}) = \left(\frac{1 \text{ oz}}{1 \text{ gallon}} \right) \left(\frac{1 \text{ gallon}}{128 \text{ oz}} \right) = 0.0078$$

3.1.2 Density/Specific Gravity (SG) Calculation

When converting v/v dilutions to w/w, the specific gravity is applied. The specific gravity of water is treated as 1.0. Obtain density or specific gravity values for the test substance from confidential statement of formula (CSF) or suitable documentation. Convert as necessary to g/mL or unitless for SG.

Conversion Example:

$$\left(\frac{9.2 \text{ lbs}}{\text{gallon}} \right) \left(\frac{1 \text{ gallon}}{3785.412 \text{ mL}} \right) \left(\frac{453.5924 \text{ g}}{1 \text{ lb}} \right) = 1.102 \text{ g/mL}$$

$$\text{Density of Product} = \frac{\text{mass (g)}}{\text{volume (mL)}}$$

$$\text{Specific Gravity} = \frac{\text{Density of Product}}{\text{Density of Water (1.0 g/mL)}}$$

$$\text{Density of Product} = 9.2 \text{ lbs/gal} \sim 1.102 \text{ g/mL}$$

$$\text{Specific Gravity} = \frac{1.102 \text{ g/mL}}{1.0 \text{ g/mL}} = 1.102$$

3.1.3 DF = (DF_{vol})(SG)

$$\text{DF} = (0.0078)(1.102) = 0.0086$$

Ecolab Controlled Document

Standard Operating Procedure

Handwritten signature and date: 11/13/18

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

3.2 Use-solution prepared per label (e.g. 1000 g use-solution prepared at 1 oz/gallon dilution):

3.2.1 Target mass (g) of product = [Total use-solution mass (g)](DF)

$$\text{Target mass (g) of product} = (1000 \text{ g})(0.0086) = 8.6 \text{ g}$$

3.2.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

$$\text{Target mass (g) of diluent} = 1000 \text{ g} - 8.6 \text{ g} = 991.4 \text{ g}$$

3.2.3 Include a range of $\pm 0.03 \text{ g}$ (~ 1 drop) or $\pm 0.3 \text{ g}$ (~ 10 drops) to target masses when preparing use-solutions.

Note: any appropriate total use-solution mass may be used.

3.3 Use-solution prepared at CSF lower certified limit (LCL) – One active ingredient

3.3.1 Determine the active ingredient concentration (ppm) in the test substance use-solution when diluted (per label or protocol) using the test substance (concentrate) with active ingredient(s) at the LCL.

Example: 1 oz/gallon

$$\% \text{ Dilution} = \left(\frac{1 \text{ oz Product}}{1 \text{ gallon}} \right) \left(\frac{1 \text{ gallons}}{128 \text{ oz}} \right) (100\%) = 0.781\%$$

$$\text{ppm active at LCL} = \left(\frac{\% \text{ Active at LCL}}{100\%} \right) \left(\frac{\% \text{ Dilution}}{100\%} \right) (\text{Specific Gravity} \times 10^6)$$

$$\text{Target mass (g) of product} = \frac{(\text{ppm Active at LCL})(\text{Total mass of use - solution})(100\%)}{(10^6)(\% \text{ Active Ingredient Result})}$$

3.3.2 Target mass (g) of diluent = [Total use-solution mass (g)] – [Target mass (g) of product]

Note: any appropriate total use-solution mass may be used.

3.4 Use-solution prepared from CSF lower certified limit (LCL) – multiple active ingredients

- Ensure that all active ingredients are at or below the calculated lower limit.
- This can be determined by calculating all active ingredient amounts and using an amount (of product) that ensures all active ingredients present to be less than or equal to the calculated lower limit.

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

3.4.1 Follow 3.3 to determine target masses (g) of product and diluent.

Note: any appropriate total use-solution mass may be used.

3.5 Determining the lower acceptance limit: The lower acceptance limit, which is the acceptable lower end of the lower limit, follows the guidance in 40 CFR Part 158.350:

If the target Lower Limit concentration (L) is (% by wt.)	Lower Acceptance Limit
$L \leq 1.0\%$	$L - 10\%$
$1.0\% < L \leq 20.0\%$	$L - 5\%$
$20.0\% < L \leq 100.0\%$	$L - 3\%$

3.5.1 Example: Product diluted at 1 oz/gallon (product/diluent) for LCL dilution use-solutions

Where: CSF LCL = 16.43%; DF = 0.0086; Nominal (N) = 17.29%

Lower Limit= (CSF LCL)(DF) = (16.43%)(0.0086) = 0.141%

Lower Acceptance Limit can be up to 5% lower or
0.141% - 0.007% = 0.134% providing a target of 0.134-0.141%.

3.5.2 For products with multiple active ingredients, the concentrate is diluted so that all active ingredients are at or below the lower limit. As a result of diluting to ensure all active ingredients present are equal to or less than the lower limit, it is possible that some active ingredients may fall below the lower end of the range. It may be acceptable if this occurs. For products with more than one A.I., when analytical methods cannot differentiate between different A.I.s in a formulation, the individual LCL values may be added together to determine the lower certified limit of the total.

3.6 Determining the upper acceptance limit:
Because of difficulties associated with generating test samples exactly at the lower limit, the EPA has provided an acceptable range above the limit, the upper acceptance limit, described in 810.2000 as follows:

If the Lower Limit concentration (L) for the ingredient is (% by wt.)	The tested value may be above the lower limit by:
$L \leq 1.0\%$	$L + 2.0\%$
$1.0\% < L \leq 20.0\%$	$L + 1.0\%$
$20.0\% < L \leq 100.0\%$	$L + 0.6\%$

Ad 10312

Standard Operating Procedure

Ecolab Controlled Document

TITLE: Test Substance Use-Solution Preparation for Analysis

NUMBER: MS088-21

- 3.6.1 Using this approach, a product that has an active ingredient with a nominal concentration of 7.00% and LCL of 6.65% (based on 40 CFR Part 158.350), would have a testing allowance of up to 6.72%. In this example, the nominal concentration is greater than 1.0% and less than 20%, therefore, the appropriate testing range would be up to 1.0% above the LCL of 6.65% ($6.65 + 0.0665 = 6.7165$, rounded to 3 significant figures = 6.72%).

4.0 RELATED FORMS

- 4.1 Form 3113: Test Substance Use-Solution Preparation for Analysis

5.0 REFERENCES

- 5.1 M032: Labeling Requirements
5.2 40 CFR 158.350
5.3 U.S. EPA OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018).

6.0 MOST RECENT REVISION SUMMARY

Added 2.1.1.1 and 2.1.1.2 and removed 2.1.2 as redundant. Clarified the purpose of using the specific gravity in 3.1.2. Revised section 3.5 and 3.6 to match current guidance used to determine the LCL. Added reference 5.3.

Prepared by: [Signature] Date: 6-11-18

Quality Assurance: [Signature] Date: 6-12-18

Management: [Signature] Date: 6-12-18

10/3/18

Form-Ver. 6016-09
Effective: 05/01/18
Form Page 1 of 1

Regulated Study Protocol Amendment

Study Title: CW32A Food Contact Sanitizing Efficacy
Study Number: 1800073
Amendment Number: 1800073-1A
Amendment Effective Date: October 10, 2018

Description of Amendment

The protocol is being amended to clarify that the test substance use-solution chemical analysis will be performed on CW32A batch P081781.

The protocol is being amended to correct the % Lactic Acid concentration listed for CW32A batch P081381 in the table on page 8. The concentration should be 33.4% resulting in 1.16 g of test substance in 598.84 grams of diluent.

The protocol is being amended to clarify why chemical analysis is being performed on lactic acid when the common practice is to only perform chemical analysis on the active ingredients. The analysis of lactic acid is being performed due to a pending decision from the EPA on whether lactic acid is an active ingredient or not. Chemical analysis will be performed on both LAS and lactic acid in the event that lactic acid is determined to be an active ingredient. Chemical analysis of the rest of the inert ingredients will not be performed.

Scientific Basis for Amendment

The protocol was amended to clarify that the test substance use-solution chemical analysis will be performed on CW32A batch P081781.

The protocol was amended to correct an error in the % Lactic Acid concentration listed for CW32A batch P081381 in the table on page 8 which affected the dilution procedure as well.

The protocol was amended to clarify why chemical analysis is being performed on lactic acid when the common practice is to only perform chemical analysis on the active ingredients.

- ☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

☒ This protocol amendment has been amended.

Refer to protocol amendment 1800073-3A for details.

Initial & Date JS 2/25/19

Laurinda Hilde
Study Director

10/10/18
Date

Page 1 of 1

Printed & Verified
Initial & Date JS 10/10/18

Regulated Study Protocol Amendment

Study Title: CW32A Food Contact Sanitizing Efficacy
Study Number: 1800073
Amendment Number: 1800073-2A
Amendment Effective Date: October 30, 2018

Description of Amendment

The protocol is being amended to change the specific gravity from 1.126 to 1.131. The change in specific gravity did not result in a change in the resulting ppm active ingredient at 0.25 oz/gallon for Dodecylbenzene Sulfonic Acid (LAS). The resulting ppm for Lactic Acid however will be amended from 648 ppm to 650 ppm. However, this change does not have an effect on the test substance use-solution dilution procedure. The use-solution acceptance criteria for Dodecylbenzene Sulfonic Acid (LAS) will remain unchanged but the use-solution acceptance criteria for Lactic Acid is being amended from 0.0583-0.0661% to 0.0585-0.0663%.

Scientific Basis for Amendment

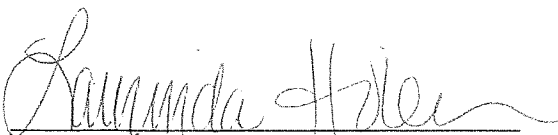
The protocol was amended to change the specific gravity from 1.126 to 1.131. The change in specific gravity did not result in a change in the resulting ppm active ingredient at 0.25 oz/gallon for Dodecylbenzene Sulfonic Acid (LAS). However, the resulting ppm for Lactic Acid did change from 648 ppm to 650 ppm. This change did not have an effect on the test substance use-solution dilution procedure or the use-solution acceptance criteria for Dodecylbenzene Sulfonic Acid (LAS). However, the use-solution acceptance criteria for Lactic Acid was changed from 0.0583-0.0661% to 0.0585-0.0663%. The change in the use-solution acceptance criteria for Lactic Acid has no effect on the study since the use-solution chemical analysis was determined to be within the new use-solution acceptance range.

- ☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

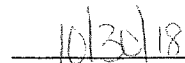
☐ This protocol amendment has been amended.


Refer to protocol amendment _____ for details.

Initial & Date _____


Study Director

Page 1 of 1


Date 10/30/18

Printed & Verified
Initial & Date  10/30/18

Regulated Study Protocol Amendment

Study Title: CW32A Food Contact Sanitizing Efficacy
Study Number: 1800073
Amendment Number: 1800073-3A
Amendment Effective Date: February 25, 2019

Description of Amendment

The protocol is being amended to include Lactic Acid as an active ingredient in addition to Dodecylbenzene Sulfonic Acid (LAS).

Scientific Basis for Amendment

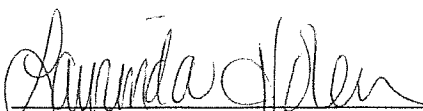
The protocol was amended to include Lactic Acid as an active ingredient in addition to Dodecylbenzene Sulfonic Acid (LAS). Efficacy testing was conducted while waiting for a decision from the EPA on whether Lactic Acid would need to be declared an active ingredient or not. The EPA has now made the decision that Lactic Acid is an active ingredient. This recent decision does not have an affect on the efficacy testing since testing was previously performed with both actives at or below their lower limits.

- ☒ This amendment does not affect the integrity of the study.
☐ This amendment does affect the integrity of the study.

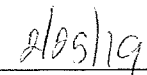
☐ This protocol amendment has been amended.

Refer to protocol amendment _____ for details.

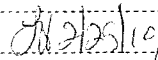
Initial & Date _____


Study Director

Page 1 of 1


Date

Printed & Verified
Initial & Date


2/25/19